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REVIEW

Conjugates of Oligonucleotides and Modified Oligonucleotides: A Review of Their Synthesis and Properties

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1. INTRODUCTION

In the molecular processes of living things, nothing surpasses Watson–Crick base pairing in importance. It is fundamental to the events that define life: the storage, transmission, and translation of genetic information. The simplicity of these hydrogen-bonded bridges and the small number of bases involved gives a predictability to the interactions of nucleic acids that is unattainable as yet with other biological molecules.

This applies even to the shortest oligonucleotides which hybridize like their larger relatives and can be viewed as informational molecules containing fragments of true genetic code. These are valuable models to investigate the physical and biological properties of DNA and RNA that would be intractable otherwise. Due to the limited number of bases used, oligonucleotides can contain the full range of functional groups and show similar chemical reactivity to true nucleic acids. This is useful for elucidation of the reactions of mutagens, carcinogens, and antitumor drugs with DNA and RNA.

As a result of their ability to base pair, oligonucleotides are used extensively in molecular biology as linkers, probes, and primers in such activities as sequencing, amplification by PCR, determination of secondary structure, engineering mutations, tailoring RNA with ribonuclease H, and assembling DNA constructs.

In other applications, "antisense" oligonucleotides can inhibit expression of viral or mRNA and, more recently, double-stranded DNA. Useful for genetic analysis, this also has potential for therapeutic application.

The recent demand for oligonucleotides resulted from improvements in their chemical synthesis that also made

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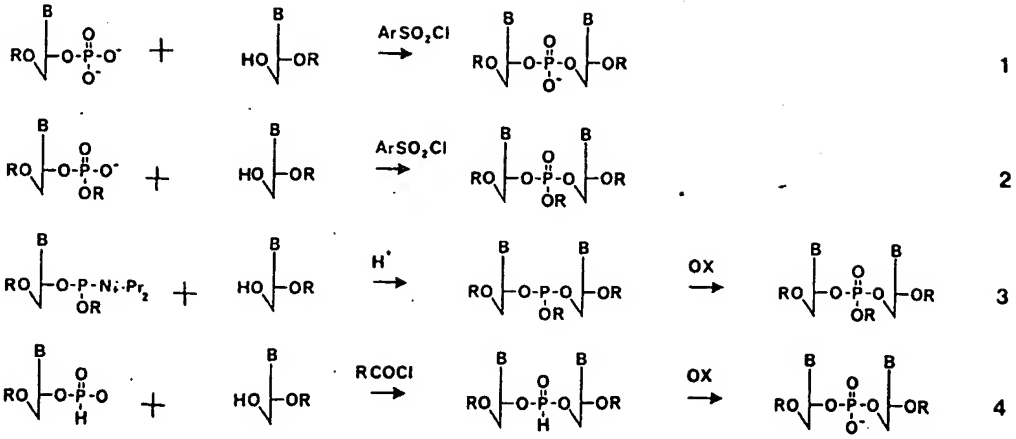
Scheme I



available a wide range of conjugates. The few, simple derivatives of dinucleotides that were available at first have grown 10 times in length and can be festooned with a plethora of different pendant groups if so desired.

Not all modifications to oligonucleotides qualify as conjugates. In this review, a conjugate is considered to result from the coupling of two or more molecules with distinct properties so that some of the characteristics of each are retained in the product. An example is the union of an oligonucleotide with a fluorescent dye to give a fluorescent oligonucleotide. A distinction is drawn between conjugates and other modifications of oligonucleotides that do not result in such a combination of properties. However, the oligonucleotide component of the conjugate may itself be modified in other ways and may carry more than one conjugate group.

Conjugates may be designed to improve some already existing feature of the oligonucleotide, for example, the strength of hybridization or uptake by cells. More often, the oligonucleotide is endowed with some completely new property, either physical or chemical, while retaining its ability to base pair. In this way, new applications have been developed that were not possible previously.

Conjugate groups combined with oligonucleotides fall into three major categories.

1. Chemically Reactive Groups. Groups that cleave or cross-link with other nucleic acids or proteins are used to study interactions between these molecules and oligonucleotides, to modify or cleave nucleic acids at particular sites, and to create possible therapeutic agents.

2. Fluorescent or Chemiluminescent Groups. These are used in nonradioactive probes or primers for automated sequencing and in physical-chemistry studies including potentially powerful new applications involving combinations of different conjugates.

3. Groups Promoting Intermolecular Interactions. The best known example is probably biotin that binds to streptavidin. This has been used particularly as a reporter system for nonradioactive probes. Other examples are intercalating agents used to strengthen the hybridization of the oligonucleotide with its complement and polylysine used to enhance cellular uptake.

This review is divided into three main parts. The first two cover the chemical synthesis of modified oligonucleotides and conjugates and the third is concerned with their properties. This includes a discussion of antisense inhibition in general terms, but applications in areas such as diagnosis or genetic analysis are not covered here and the reader is referred to other sources.

2. CHEMICAL SYNTHESIS OF OLIGONUCLEOTIDES

Much of the chemistry described in this review has been developed around the methods for synthesizing oligonucleotides. These are introduced in section A only in sufficient detail to provide the necessary background for what follows.

The oligonucleotide component of a conjugate may contain all natural nucleotides or may itself be modified. Section B reviews methods for the synthesis of modified oligonucleotides.

A. Strategies of Synthesis. The impetus for the current interest in oligonucleotides derives from developments in two areas. Advances in molecular biology over the last decade or so created uses for these compounds while advances in chemical synthesis made them available for practical applications. A synthesis that took man-years of work in 1979 (*1*) could be done today in a few man-hours.

This resulted from improvements in two aspects of oligonucleotide synthesis. One was the development of solid supports that made possible the automation of the process and led to microprocessor-controlled synthesizers. The other was improvement in the synthesis of phosphate esters to give the coupling efficiencies of 98% and above that are necessary to take full advantage of the benefits that solid supports offer. The historical development of reactions used for nucleotide coupling is indicated in Scheme I and fuller accounts of oligonucleotide synthesis may be found in refs 2–5.

The first of these reactions is called the diester approach and was developed by Khorana (*1*). Ester formation was effected with dicyclohexylcarbodiimide or an aryl sulfonyl chloride. This coupling reaction is no longer used, but despite some shortcomings, the amide protecting groups developed for the bases and the dimethoxytrityl for 5′-OH are still standard.

This synthesis was improved by protecting the phosphate group to give the triester approach in reaction 2. This not only prevented side reactions but greatly facilitated workup and purification by enabling much larger scale and more rapid purification by chromatography on silica gel in organic solvents. With improved coupling reagents such as sulfonyl tetrazolides, this method was sufficiently efficient for solid support synthesis and is still used to some extent today (*6*).

The next improvement was to replace the reacting phosphate with a trivalent phosphite. Originally, a phosphorochloridite was used (*7*) but now the phosphoramidite in reaction 3 is preferred (*8*). Activation is by a mild

proton donor, usually triazole, and after each coupling reaction the phosphite is oxidized to the pentavalent level. This is the route employed most often today and permits synthesis of chain lengths considerably in excess of 100.

The fourth and most recent method (*9*, *10*) returns to pentavalent phosphorus, this time a hydrogen phosphonate after which this approach is named and which was first recognized as a potential synthon some time ago (*11*). A carboxylic acid chloride is used as condensing agent. This method is comparable in efficiency to the previous one although it is generally considered inferior for very long sequences. It has the advantage that the oxidation step does not have to be performed after each coupling reaction but can be left until the end of the synthesis. This simplifies and speeds up the process but, more importantly, it facilitates the synthesis of modified oligonucleotides where sulfur, nitrogen, or other elements replace oxygen in the reaction at phosphorus.

These synthetic approaches have been adapted for use in the ribo series (*12–14*) and for nucleosides with unnatural L-sugars (*15*) or α-glycosidic linkages (*16*).

B. Synthesis of Modified Oligonucleotides. This section reviews some of the options that are available for modification of the oligonucleotide component within the conjugate. The consequences of these changes for biochemical properties are discussed in section 3.

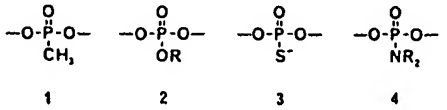
Modifications to the oligonucleotide have been employed most often for use in antisense inhibition where it is necessary for oligonucleotides to survive in cell cultures or other biological environments and also to cross the cell membrane. Nucleases are widespread and the lipophilic cell membrane is an effective barrier against passive diffusion of polyelectrolytes.

Often, modifications can be chosen to improve performance in both areas. For example, increasing lipophilicity to improve uptake is likely to decrease the rate of degradation by nucleases that are designed to degrade polyanions. The conjugate group itself may prove beneficial in these regards if it is lipophilic or otherwise inhibits the action of nucleases. As yet, the number of examples of conjugate groups combined with other modifications to the oligonucleotides is small but increasing rapidly.

Changes might be made at the bases, the sugars, the ends of the chain, or at the phosphate groups of the backbone. Those at the bases and sugars are generally the most difficult chemically and require the greatest amount of synthesis. In addition, they must not disrupt the ability of bases to form hydrogen bonds. One example is the use of α-nucleotides discussed later.

Modifications to the ends and backbone of the molecule are easier synthetically. Because the 5′-terminus is the most common site for conjugation, the phosphate groups are often available for further derivatization. As they are the site of action of nucleases and also carry the charges that inhibit cellular uptake, this presents the most direct approach to improvement in these areas.

The type of phosphate modification most studied is to replace or block the negatively charged oxygen atom to give structures 1–4. The syntheses of these and other modifications are discussed below.



1 2 3 4

(*i*) *Methylphosphonates* (*1*). These nonionic derivatives were introduced by Miller et al. in 1979 (*17*) and are one of the most extensively studied classes of oligonucleotides because of their useful chemical and biological properties. Older methods for their synthesis were based on modifications of the triester approach (*17–23*). Following the introduction of phosphine reagents (*24–26*), solid-support synthesis based on phosphoramidite chemistry has become standard (*27*). Phosphonamidite reagents react as efficiently as the usual phosphoramidites and can be used interchangeably during automated synthesis to insert the uncharged linkage at any or all positions within the sequence. Products from this approach were found to give better melting curves than those from a synthesis based on triester type chemistry (*28*).

Because of the different nature of the backbone, special methods for their characterization have been developed (*29*). Like purification, this is still not as easy as with fully charged phosphodiesters.

Only the methyl substituent on phosphorus has been investigated to any extent. A phenyl phosphonate was prepared early on (*18*) and a 4,4′-dimethoxytriphenylmethanephosphonate was obtained unexpectedly from an attempted Arbusov reaction (*30*). More recently, a (difluoromethyl)phosphonate was synthesized to mimic the polarity of the natural oxygen atom more closely (*31*).

(*ii*) *Phosphotriesters* (*2*). These compounds were used as nonionic analogues of oligonucleotides by Miller et al. before the methylphosphonates (*32*). They are more difficult to synthesize and, as a result, most studies have been limited to oligonucleotides containing only a single triester or only thymine bases.

The problem is the lability of the triester function during the basic conditions used in deblocking or cleaving from the solid support. Use of milder conditions (*32*, *33*) or more labile amine protecting groups (*34–37*) or more stable triesters (*38*, *39*) have not yet given a method as versatile as synthesis of the phosphonates.

Originally, methanol or ethanol in the presence of tosyl chloride was used to esterify internucleoside phosphates after each coupling in short sequences (*32*, *40*, *41*). Recently, it has been reported that this reagent can give complete triesterification of heterosequences as long as 10–20 bases following temporary protection of the amino groups with 9-fluorenylmethoxycarbonyl chloride (9-fluorenylmethyl carbonochloridate) in solution (*36*). This is the only method that gives extensively esterified products of this size. Methyl methanesulfonate has been used also as a methylating agent (*42*).

Different triesters have been introduced as the protecting groups during synthesis by the triester (*43*), phosphite (*38*, *39*), or amidite (*33*, *34*, *37*, *44*) approaches or by oxidation of hydrogen phosphonates with alcohols (*45*). In addition, transesterification in the presence of fluoride ions can replace aryl protecting groups on phosphate by alkyl to give more stable esters (*41*, *46–48*).

Groups that have been used other than the usual methyl and ethyl include 1,1-dimethyl-2,2,2-trichloroethyl (*38*, *39*, *43*), isopropyl (*33*), neopentyl (*48*, *49*), *n*-butyl (*45*), and 2,2,2-trifluoroethyl (*37*).

(*iii*) *Phosphorothioates* (*3*). Used extensively by Eckstein and his co-workers for the study of enzyme mechanisms, these derivatives are the closest to the natural nucleic acids in terms of structure and charge density. For reviews of their preparation and uses see refs 50–52. Both DNA and RNA polymerases accept the appropriate 5′-*O*-(1-thiotriphosphates) as substrates to give products containing just the R_p stereoisomers of phosphorus.

Chemical synthesis is also straightforward. Eck-

stein's use of elemental sulfur to oxidize dinucleoside phosphites is applicable to phosphoramidite synthesis on solid supports giving oligonucleotides with phosphorothioate linkages throughout or just at selected positions (*50, 53, 54*). A concern is that the phosphorothioate functions are subjected to an iodine oxidation step after each subsequent round of coupling which could lead to replacement of some sulfur by oxygen (55). In practice, phosphorothioate could still be isolated after 48 rounds of coupling (*53*) and NMR studies indicated that about one sulfur atom per molecule was lost to oxidation during the synthesis of a 20-mer (*56*).

Elemental sulfur can also be used for oxidation following the hydrogen phosphonate approach (*45, 57–61*). This offers some practical advantages over the phosphoramidite method where oxidation has to be performed after each coupling. The usual solvent for sulfur is carbon disulfide, which is volatile, malodorous, and troublesome in automated synthesizers, where the sulfur tends to form solid deposits. It is preferable to perform the oxidation just once manually at the end of the synthesis.

For synthesis of chimeric structures containing both phosphorothioates and phosphodiesters in the backbone, treatment with sulfur partway through the synthesis is followed by further rounds of coupling and oxidation. This proved more successful with the phosphoramidite than the hydrogen phosphonate method. Unprotected phosphorothioates generated on sulfur treatment of hydrogen phosphonates survived subsequent coupling and oxidation cycles much less well than the blocked intermediates formed from phosphites (*60*).

(*iv*) *Phosphoramidates* (*4*). An appealing feature of phosphoramidates is the diverse range of amines that might be introduced. Given a good, general synthesis, this class of compounds should offer the greatest opportunity for structural variation. Substituents on the amine could include conjugate as well as nonconjugate groups. It might be desired to introduce just one phosphoramidate at a specific position or to modify all the phosphates with either the same or different substituents. Such precision and control is attainable with current synthetic methods.

A number of reactions for preparing phosphoramidates have been applied to oligonucleotides with greater or less success as discussed in ref 62. The prefered method uses an amine in the presence of carbon tetrachloride or iodine as the oxidant in the hydrogen phosphonate approach (*45*). By leaving this step until the end of the synthesis, phosphoramidate linkages are introduced throughout the sequence (*15, 58, 63, 64*). Alternatively, further rounds of coupling can be performed after amidation to generate a heterogeneous backbone (*45, 61, 64*). By alternating hydrogen phosphonate coupling steps with phosphoramidite, various unmodified and modified linkages can be inserted at specific sites throughout the sequence (*61, 63*).

Another attractive feature of phosphoramidates is that they may be readily converted to phosphodiesters for characterization by the usual means (*45, 64*).

The P–N bond is hydrolyzed in acid and, unless the nitrogen is substituted, it is also too base labile to survive the deblocking reaction (*64, 65*). This bond is more labile in the ribo series (*65*). The oligonucleotide amidates made by this method are listed in Table I.

(*v*) *Other Phosphate Modifications*. The four types of phosphate modification discussed above have all been used fairly extensively to modify the properties of oligonucleotides or their conjugates. Other modifications that have been made but, for the most part, not yet studied in this way are listed in Table II. Further examples include derivatives with pentavalent phosphorus (*80, 81*), but many more possibilities remain unsynthesized.

Table I. Amidates Made by the Hydrogen Phosphonate Approach

structure of the amine component	refs	structure of the amine component	refs
$NHCH_3$	45, 64	$NHCH_2CH_2$-morpholinyl	63
NHC_4H_9	45, 58, 62	$NH(CH_2)_2NHCO$-cholesteryl	61
NHC_8H_{17}	62		
$NHC_{12}H_{25}$	62	piperidinyl	45
$NH(CH_2)_2OCH_3$	64	morpholinyl	45, 58, 64
$N(CH_3)_2$	62, 64	piperazinyl	58
$N(CH_3)CH_2CH_2N(CH_3)_2$	63	*N*-methylpiperazinyl	45

Table II. Modified Internucleoside Phosphates

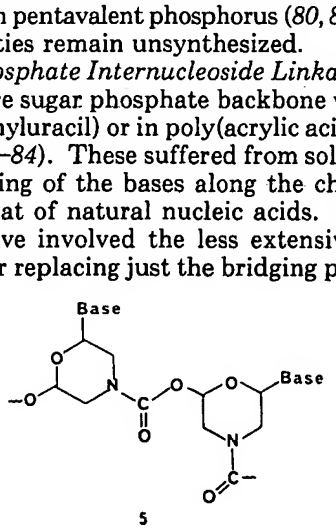
A	B	C	D	refs
NH	O	O	O	66, 67
O	O	NH	O	67, 68
O	S	O	CH_3	69
O	O	O	Se	53, 70
O	O	S	O	71
O	NPr	O	NEt_2	72
O	S	O	NEt_2	72
O	Se	O	NEt_2	72
O	S	O	CH_3	73
O	S	O	S	74
O	S	O	NHR	74
O	S	O	OPr	33, 75
O	S	O	OEt	44, 76
S	O	O	O	77
O	O	OPO_3	O	67
CH_2	O	O	O	78
S	O	O	O	79
S	S	O	O	79
O	O	S	CH_3	267

Table III. Groups Used To Replace Internucleoside Phosphates

group	refs
-OCOO-	85–88
$-OCH_2CONH-$	89
$-OCH_2COO-$	90
-OCONH-	89, 91–93
$-OSiR_2O-$	94–96

Further examples include derivatives with pentavalent phosphorus (*80, 81*), but many more possibilities remain unsynthesized.

(*vi*) *Non-Phosphate Internucleoside Linkages*. In early work, the entire sugar phosphate backbone was replaced as in poly(1-vinyluracil) or in poly(acrylic acid) hydrazide derivatives (*82–84*). These suffered from solubility problems and spacing of the bases along the chain was different from that of natural nucleic acids. Most recent approaches have involved the less extensive modification in 5 (*97*) or replacing just the bridging phosphate by



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the groups in Table III. As yet, synthesis of these types of compound is still at an early stage. Solubility in water is a common problem and hybridization is different from that of natural oligonucleotides in some cases.

3. SYNTHESIS OF CONJUGATES

Conjugate groups may be coupled to oligonucleotides either through sites present naturally in nucleic acids or through some other reactive linker group introduced specifically for the purpose. The naturally occurring groups that can be used are amino groups on the bases, hydroxyl groups on the sugars, and phosphate groups, both terminal and internal. Linker groups attached to the oligonucleotide for derivatization are most commonly primary amines, thiols, or aldehydes, but the possibilities are many. Often, the linker is attached to the oligonucleotide by a spacer arm either to facilitate coupling or to distance the conjugate group from the oligonucleotide.

Either the conjugate group or the linker may be introduced at one of three stages during oligonucleotide synthesis. These each have advantages and disadvantages.

1. The group can be attached to a nucleotide before incorporation into the growing chain. This approach can be used for either chemical or enzymatic synthesis of oligonucleotides.
2. Molecules other than nucleotides can be introduced during the synthesis of the oligonucleotide.
3. A linker or conjugate group can be attached to a natural nucleic acid or to a synthetic oligonucleotide after deblocking.

Sections A and B below describe incorporation during chemical or enzymatic synthesis, respectively. Section C covers coupling following synthesis and section D lists some conjugate groups that have been used.

A. Incorporation of Conjugate and Linker Groups during Chemical Synthesis of Oligonucleotides. From a synthetic point of view, incorporation of conjugates and linkers during the assembly of an oligonucleotide rather than afterward is the most rigorous approach. It gives greatest control over the number and location of the modifications; side reactions are minimized by the protecting groups on the nucleotides, and advantage is taken of the benefits of solid-support synthesis for workup and purification. Two strategies will be considered in which the conjugate group or linker may or may not be part of a nucleotide synthon. In both cases, the reactions are performed under anhydrous conditions, unlike the postsynthesis modifications described later, which are performed largely in aqueous solution.

(*i*) *Incorporation of Modified Nucleotides.* This approach is appealing for chemical synthesis as the nucleotide building block carrying the desired modifier can be introduced precisely at any internal or terminal position in the oligonucleotide with assurance that modification at that position is complete. As a result, concerns over the uniformity of the product and its identity should be less than those with some other methods. However, it is necessary to first prepare and purify the nucleotide, and the modifications must be able to withstand the coupling reaction and the rigors of acid and basic deblocking. In some cases, nonstandard protecting groups may be necessary. Probably as a result of the greater synthetic effort required, this approach has not been used as widely as others.

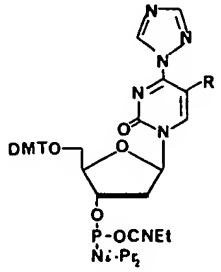
Substituents may be attached to nucleotides at the base, sugar, or phosphate residues, but ideally, changes should not interfere with hybridization. While there is potential for using phosphate-modified precursors such as substituted phosphonates or phosphotriesters, little work has been done in this regard other than with the simple blocking groups described in the previous section.

Two sites on bases which are easy to manipulate chemically without necessarily preventing base pairing are C(5) of uracil and N(4) of cytosine, and several nucleotide phosphoramidite synthons have been prepared with protected linkers at these positions (Table IV).

Table IV. Linkers Incorporated into Oligonucleotides as Substituents on Pyrimidine Nucleoside Phosphoramidate Synthons

substituent	refs
At N(4) of dC	
$(CH_2)_6OH$	98
$(CH_2)NHCO(CH_2)_5NH_2$	99
At C(5) of dU	
$(CH_2)_3NH_2$	100–102
$C{=}C(CH_2)_2NH_2$	103
$C{=}CCH_2NHCO(CH_2)_5NH_2$	103
$CH{=}CHCONH(CH_2)_6NH_2$	104

The triazole leaving group introduced with reagent **6** can be displaced by various nucleophiles before the final



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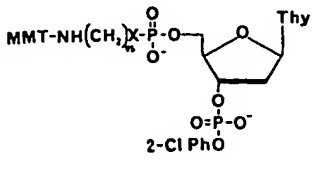
deblocking of the oligonucleotide (*105–107*). This might be used as a general-purpose reagent to minimize the synthetic effort necessary to introduce a variety of other groups or for the introduction of a group sensitive to the coupling or acidic detritylation steps.

In addition, a number of nucleotides already bearing conjugate groups at these positions have been used. Examples are EDTA, used in the generation of free radicals (*108*), and biotinyl, dinitrophenyl, pyrenyl, and dansyl reporter groups (*109*). 5-Bromo-2′-deoxyuridine has been incorporated by triester synthesis for its ability to cross-link with DNA-binding proteins on UV irradiation (*111*).

In the case of purines, C(8) of adenine was used as an attachment site for the photoactivatable cross-linking reagent psoralen (*112*).

Other nucleotide phosphoramidites have been prepared with O(5′) of the sugar replaced by nitrogen or sulfur (*113–115*). Used in the final coupling, these give oligonucleotides with a thiol or primary amine at the 5′-end for subsequent reaction with electrophiles. For situations where a spacer was required between the 5′-amino and the oligonucleotide, nucleotide **7** was used with triester coupling (*116*).



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Also in the triester series, protected thymidine 3′,5′-diphosphate was used for the introduction of a terminal 5′-phosphate residue as a site for postsynthetic modification (*117*).

Unlike with solid-support synthesis, with the older solution-phase triester method nucleotides can be added to the 3′-end of the oligonucleotide. Nucleotides carrying

various 3′-bound conjugate groups or linkers have been introduced this way (*117–120*).

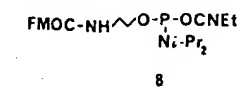
To accomplish this by solid-support synthesis, a special support bearing the modified nucleoside may be prepared as in the case of cytidine with an amine linker at N(4) (*121*).

(*ii*) *Incorporation of Non-Nucleotides.* A number of reagents other than nucleotides have been developed for incorporation during oligonucleotide synthesis. These are generally much simpler synthetic targets for the introduction of conjugates or linkers. They are used most commonly to couple a group at the 5′-end of an oligonucleotide while it is still fixed to the solid support. This reduces the potential for side reactions at the bases, which are still protected, and simplifies purification. The reagent must be soluble in an organic solvent suitable for the coupling reaction and, again, must withstand the deblocking procedure.

Most groups introduced in this way have been used to give, after deblocking, a nucleophilic linker for preparation of a conjugate. One reason for using nucleophiles is that many suitable electrophilic derivatives of the conjugate groups are available commercially.

Often, the same coupling chemistry is used for nucleotides and non-nucleotides so that the latter may be added from a spare reservoir of the synthesizer using the standard program.

Reagents 8–11 have a protected amine, thiol, or carboxyl at one end of a spacer and a phosphoramidite at the other (*122–128*). Reagent 12 is a cyclic analogue of



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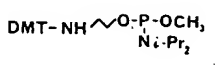

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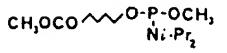

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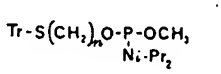

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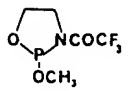

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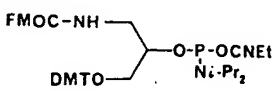

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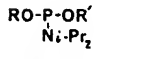

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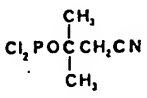

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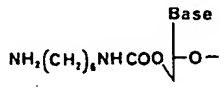
9 that uses the same nitrogen atom both as the leaving group and as the linker (*129*). These compounds can be used interchangeably with the standard nucleoside phosphoramidites. Analogous reagents are available for H-phosphonate (*130*) or triester synthesis (*120, 131*) or linking through a methylphosphonate (*132*). Hence, these groups can be incorporated readily during automated synthesis, whichever approach is employed, without the necessity for changing reagents. After deblocking, all give oligonucleotides with a nucleophilic linker at the 5′-end. Reagent 13, containing an additional protected hydroxyl function, can be used for multiple rounds of deblocking and coupling to increase the number of linker groups (*153*).

Phosphoramidite derivatives of biotin (*133*), acridine (*134*), and anthraquinone (*135*) have been use similarly and phosphate derivatives of acridine and tetramethylrhodamine have been used for triester synthesis both by solid-support and solution-phase synthesis (*131, 136*).

During automated synthesis, reagents of general structure 14 or 15 can be used to phosphorylate or thiophosphorylate the 5′-hydroxyl group (*118, 137–140*) and a number of other reagents exist based on triester chemistry (*141* and references therein). The phosphate can be used for modification at a later stage or it can be selectively deblocked before cleaving from the solid support and condensed, for example, with an alcohol in the presence of a sulfonyl triazolide (*142*).

As an alternative to reactions at phosphorus, the 5′-hydroxyl group of support-bound oligonucleotide can be activated with carbonyldiimidazole for reaction with hexamethylenediamine to give carbamate 16 (*143*). Described

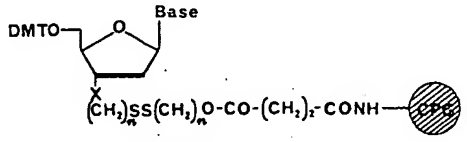


16

in section C below is the use of a vicinal diol as a linker. This can be introduced as a ribonucleotide joined 5′–5′ during the final coupling of the synthesis (*122*).

Modifications at the 3′-end are less common due to the inaccessibility of this site in solid-support synthesis. The usual way to solve this problem is to use a support with the modification already built in. For example, polyamide or polypeptide chains synthesized on a support were used to initiate oligonucleotide synthesis to give conjugates with a 3′-polypeptide tail. Incorporation of lysine in the peptide furnished multiple amino linker sites for subsequent derivatization (*144, 145*).

The spacer between the nucleoside and the support can be designed so that on cleavage, a reactive nucleophile is generated at the end of the oligonucleotide. For example, support 17 gave a 3′-terminal thiol after ammo-



X = S, ArOPO

17

nia treatment followed by reduction (*146, 147*). To avoid the need for four such supports, a linker was developed to which the first nucleoside was added during the synthesis. On cleavage, this liberated a 3′-terminal amine from a carbamate (*148*).

Reactions at the 3′-end may be performed more readily during solution-phase synthesis. Examples are the condensation between a 3′-phosphate and the hydroxyl group of an acridine or phenanthroline derivative using triester chemistry (*136, 149, 150*).

A site amenable to ready modification during oligonucleotide synthesis is the internucleoside phosphate, particularly during the oxidation of intermediate phosphites or hydrogen phosphonates. Much of the work on conjugation here has been done by Letsinger and his co-workers, who have reported several methods for linking through phosphoramidates to prepare conjugates of phenanthridine and cholesterol (*43, 61, 151*). Other workers have introduced acridine by triesterification of unprotected internucleoside phosphates (*136*). Of these approaches, the most routine and widely applicable would seem to be generation of phosphoramidates during hydrogen phos-

Table V. Linker and Conjugate Groups Incorporated Enzymatically into Oligonucleotides as Substituents on Nucleotide Triphosphates

substituent	refs
At C(5) of (d)UTP	
naphthalene derivative	102
biotin	154–157
$(CH_2)_{12}NH_2$	157
$CH{=}CHCH_2NH_2$	157
N_3[a]	158, 159
fluorescein	160
SCH_3[b]	161
At N(4) of (d)CTP	
$(CH_2)_6NH_2$	162
At C(4) of UTP	
S[c]	163, 164
At C(8) of ATP	
$NH(CH_2)_6NH_2$	157
2,4-dinitrobenzene[d]	165
At N(6) of ATP	
$CH_2CONH(CH_2)_6NH_2$	157

[a] Used to cross-link with proteins. [b] Used in site-specific cleavage of DNA. [c] Used as a site for alkylation. [d] Used as a hapten.

phonate synthesis. This has been used to introduce a protected amino linker for postsynthetic derivatization (*152*).

Another easily accessible phosphate derivative is the phosphorothioate described previously. The sulfur atom reacts readily with alkylating reagents as will be discussed in section C.

Finally, the versatile trifunctional phosphoramidite 13 can be used in place of a nucleotide during synthesis to introduce one or more amine linkers into the backbone at any position (*153*).

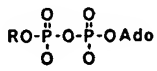
B. Incorporation of Modified Nucleotides during Enzymatic Synthesis of Oligonucleotides. The strategy of incorporating modified nucleotides can be applied to enzymatic synthesis of oligo- or polynucleotides with or without a template. While the products are not subjected to the conditions of chemical synthesis and deblocking, this method generally gives less control over the sites of modification and is restricted by what is acceptable as a substrate for the enzyme.

A variety of DNA and RNA polymerases have been used for this purpose, but those most commonly employed are *Escherichia coli* DNA polymerase I and terminal deoxynucleotidyl transferase. The former is used with a template either for internal incorporation of a modified nucleotide or for addition to the end of a presynthesized oligonucleotide. The latter enzyme is used without a template to add one or more nucleotides to the 3′-end.

Generally, the same positions on the nucleotides are modified as for the chemical syntheses discussed previously. These are C(4) and C(5) of pyrimidines and C(8) of purines and examples are given in Table V. Some of the more commonly used nucleoside triphosphates are commercially available.

T4 RNA ligase has been used to introduce a single, modified nucleotide at the 3′-end of RNA or DNA terminating with a ribonucleotide. The 3′-thiophosphoryl derivative of pCp gave the same phosphorothioate function whose chemical synthesis was described previously. Also incorporated was the fluorescent derivative with bimane attached to sulfur (*166*).

In the absence of ATP, T4 RNA ligase transfer the *non-nucleotide*-bearing phosphate from the ADP derivative 18 to the 3′-hydroxyl of an RNA (*167*). This has been used to label RNA with various reporter molecules (*168*).



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C. Postsynthetic Modifications of Oligonucleotides. Conjugate groups are usually introduced after the synthesis and deblocking of the oligonucleotide. This normally requires less effort than preparation of reagents for incorporation during synthesis but introduces other problems.

As oligonucleotides are polyionic, postsynthetic reactions are usually performed in water or an aqueous solvent in which the reagents must be sufficiently soluble and stable. This, in itself, is restricting as few synthetic, organic reactions are intended to be performed under these conditions.

As linkers are usually nucleophiles, unwanted reactions may occur at many internal sites in oligonucleotides. The separation of oligonucleotides with different numbers of conjugate groups is difficult as is their characterization, which is usually not attempted in any rigorous way and often not at all. The structure of the product is frequently assumed from the nature of the starting materials, particularly with conjugates of large, multifunctional molecules, where it is generally not possible to apply the more rigorous standards of organic chemistry. However, the reasons for making these compounds are usually for practical applications and, provided that they function as intended, then the precise number and location of the conjugate groups may not be crucial.

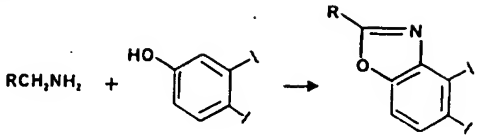
Postsynthetic reactions on oligonucleotides may be used to generate or modify linkers or to introduce the conjugate group. This section describes, in rather broad terms, the types of reactions that have been employed with various linker functions. The emphasis is on the nature of the bond-making reactions rather than particulars of individual cases. Subsections are devoted to coupling reactions used for different linker groups.

(*i*) *Reactions of Primary Alkylamines.* Primary alkylamines (and hydrazines) are among the most commonly used linkers because of their affinity for electrophiles. Usually, these are some activated form of a carboxylic acid such as the ester of *N*-hydroxysuccinimide that gives an amide (*99*, *100*, *121*, *122*, *124*, *130*, *169–171*). Other related species that have been used include nitrophenyl (*126*, *151*, *172*) and pentachlorophenyl esters (*115*), an acid anhydride (*102*, *173*, *174*), and sulfonyl chlorides (*99*, *102*). Alternatively, the water-soluble 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide condensing reagent (EDC) can be used for amide formation with a carboxylic acid (*170*, *175*). Formally, this reagent accomplishes a dehydration reaction in water.

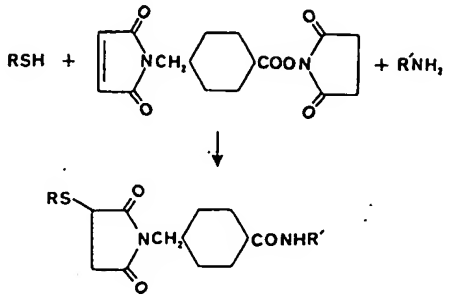
It was found with EDC that as many as 35–45% of the amide bonds formed were with the amines of the bases rather than with the intended linker. The *N*-hydroxysuccinimide ester gave less reaction at the bases but was also less efficient overall (*170*). This study was of reactions between oligonucleotides and carboxyl groups attached to solid matrixes, but extensive base modification was found also on EDC treatment of an oligonucleotide bearing a 5′-carboxyl group (*127*).

Chief among the reagents giving non-amide products are isothiocyanates that give thioureas (*99*, *113*, *122*, *130*). Other reactions that have been used are hydrazone for-

Scheme II



Scheme III



mation with an aldehyde (*176*) and a less general reaction with nitrobenzodiazole fluoride (*113, 122, 130*). The unusual reaction in Scheme II brought about by horseradish peroxidase in the presence of hydrogen peroxide was used to introduce an intercalating agent (*177*).

A molecular adapter has been designed that will replace an amino linker with a thiol (*121*) while bromopyruvate was used to couple an amino linker with a thiol in a protein (*178*).

(*ii*) *Reactions of Thiols.* The chemistry of sulfur is more complex than that of nitrogen, and thiol linkers may be used in a greater variety of reactions than amines. As well as their nucleophilic properties, they bond readily to mercury and form disulfides with other thiols. In fact, disulfide formation can be a problem on storage (*127*).

In order to generate conjugates from two thiols by mixed disulfide formation, competing reactions to give symmetrical products must be suppressed. This is usually done by first forming a mixed disulfide between one thiol and 2-thiopyridine followed by an exchange reaction with the second thiol (*127, 146, 179, 180*). Alternatively, one of the thiols may be used in large excess (*181*).

A useful feature of disulfide formation is that it is readily reversed by treatment with dithiothreitol (DTT) or other mercaptans. This has been used to remove one-half of the conjugate after it has fulfilled its function (*181*) or to reversibly bind oligonucleotides to a solid matrix (*127*). Reversal of disulfide formation was used to generate thiol linkers from blocked disulfide precursors (*121, 146, 179*).

The most commonly used electrophilic groups for conjugation with thiol linkers are iodo- or bromoacetates and maleiimides (*121, 123, 130, 131*), but some fluorescent markers proved troublesome by this approach (*130*). Immobilization on a solid support was claimed to be far superior with thiol as opposed to carboxyl or amine linkers (*127*).

By a type of Michael addition, the thiol on an oligonucleotide was linked with an amine in a one-pot reaction using the bifunctional reagent in Scheme III (*182*).

The affinity of thiols for mercury compounds was used for immobilization of an oligonucleotide on chloromercuribenzoate-derivatized agarose (*127*).

Another finding reflecting the more complex chemistry of sulfur is that a two-carbon spacer between sulfur and phosphate was unstable at pH 8 while longer ones were not (*123, 146*).

(*iii*) *Reactions of Phosphates and Thiophosphates.* Terminal phosphates may be introduced during chemical synthesis, by enzymic phosphorylation or by β-elimination of a terminal ribonucleoside following periodate oxidation. Naturally derived material may already have a terminal phosphate. The only reactions used to form conjugates postsynthetically at phosphate are condensations with amines or alcohols to give phosphoramidates and esters. Thiophosphates are used in a different class of reactions with alkylating agents.

An early method from Khorana's laboratory for reaction at terminal phosphates was the formation of a phosphoramidate from aniline using dicyclohexylcarbodiimide (DCC) as the condensing reagent in a mixed solvent of water, dimethylformamide, and butanol (*183*). This is still basically the method used to modify oligonucleotides except that DCC has been replaced by water-soluble EDC (*67, 184–186*). Recently, cyanogen bromide has been used for the same purpose. This reagent may also be used to generate a variety of unusual internucleoside phosphate bridges by coupling contiguous oligonucleotides on a template in a form of chemical ligation (*67, 185, 186*).

Although they were not the first to use EDC with oligonucleotides, Chu et al. developed the protocol most commonly used of first preparing the phosphorimidazolide with EDC and reacting this with the amine or other nucleophile (*187*). This intermediate, used previously in chemical ligation (*188*), is faster than direct coupling of simple amines and permits the use of nucleophiles that would themselves react with EDC. Although side reactions at the bases do occur, they were considered to be minor under the conditions used. Other workers have reported that *N*-hydroxybenzotriazole is superior to imidazole for this purpose (*67*).

This approach has been used to attach a variety of linkers and conjugate groups to oligonucleotides (*169, 170, 173, 179*). Direct coupling to the amine without going through the intermediate phosphorimidazolide has been used also (*67, 189*). The reaction has been extended to nucleophiles other than amines and has been studied in some detail to minimize base modification (*184*).

Thiophosphate may be introduced during chemical synthesis or with polynucleotide kinase (*190*). The sulfur atoms at both terminal and internucleotide sites react at least 10^3 times faster than the other groups in nucleic acids with 2-chloroethylamines and so can be used for selective coupling (*190*). A variety of alkylating agents have been used to form conjugates in this way (*118, 140, 150, 166, 191–194*).

(*iv*) *Electrophilic Linkers.* Most examples of conjugate formation use a nucleophilic linker to react with some electrophilic reagent. Unwanted reactions may occur at other sites in nucleic acids which are predominantly nucleophilic in nature, particularly the bases. An electrophilic linker may be used providing that it does not undergo intramolecular coupling at these sites. There are a few examples of this approach, the electrophiles being carbonyl or activated carboxyl groups.

One of the earliest methods for labeling RNA with biotin used periodate to oxidize the 3′-terminal ribonucleoside to a dialdehyde that was reacted with a primary amine and reduced with borohydride (*195*). This has been used to label RNA with a number of fluorescent dyes and other agents (*196–198*). In the case of oligodeoxynucleotides, a terminal ribonucleotide can be introduced by 5′–5′ coupling as the last step in synthesis (*122*). Steric hinderance around the reacting amino group may be troublesome in some cases with this method.

A single aldehyde function can be generated at the 5′-end by periodate oxidation of a vicinal diol introduced during synthesis. Reaction with an amine or hydrazine followed by reduction under very mild conditions did not show any competing side reactions and was particularly good for immobilization on a solid support (*125*).

Another electrophilic species was generated at the 5′-terminus by treatment of a carboxyl group with EDC in the presence of imidazole. This was used for amide formation (*125*).

(*v*) *Reactions at Naturally Occurring Sites in Nucleic Acids.* Unmodified oligonucleotides contain a number of reactive sites that are generally less potent than the synthetic linkers but which have been used for conjugation. For the most part, these are nucleophiles and include nitrogen functions on the bases, C(5) of pyrimidines, C(8) of purines, primary and secondary hydroxyl groups, and phosphomono- and diesters. Electrophilic substitution can also occur at some positions on the bases. Reactions at these sites are likely to lead to complex mixtures unless they can be controlled in some way. This may be acceptable for labeling of large DNA or RNA, where modification can be restricted to a very small fraction of nucleotides and the level of heterogeneity in the product is undetectable and irrelevant. The approach is less satisfactory for oligonucleotides where incomplete modification gives products with a spectrum of properties. Four of these reactions that are better defined chemically are given below.

The first uses a photoactivatable derivative of biotin carrying a phenyl azide that reacts with nucleic acid bases (*199*). This is used to label about only 1 in 50 bases in DNA so as not to impair hybridization and may therefore not be applicable to oligonucleotides.

In a second method, mercury at C(5) of pyrimidines, introduced by reaction with mercuric nitrate, was coupled with haptens bearing thiol groups (*200*).

Bisulfite adds across the 4,5-double bond of cytidine and encourages nucleophilic displacement of the amino group at C(4). This reaction is selective for single-stranded nucleic acids and has been used to introduce a number of substituents (*178*, *201*, *202*).

Finally, an electrophilic substitution occurs at C(8) of guanine in nucleic acids on reaction with *N*-acetoxy-2-aminofluorene to give a label for immunological detection (*203*, *204*).

None of the above reactions can be used to modify an oligonucleotide in a controlled way other than by restricting to one or two the number of reacting nucleotides, thereby severely limiting the sequence. There are few examples where a more controlled approach is possible.

4-Thiouridine is a naturally occurring nucleoside that can be incorporated into oligonucleotides. It was shown that alkylation with α-haloacetamido derivatives or phenacyl bromides could be directed exclusively to the sulfur of this base to introduce a number of different conjugate groups (*163*, *164*).

An example of a reaction restricted to a particular sequence involves introduction of psoralen onto thymidine only when it is flanked on the 3′-side by adenosine (*205*). This was possible because, on irradiation, psoralens preferentially cross-link double-stranded oligonucleotides at thymidine in the sequence 5′-TpA-3′. The reaction can be partially reversed on irradiation at a different wavelength to give, after strand separation, a single strand with the psoralen monoadduct of thymidine. This serves as a reactive probe that cross-links with its complementary sequence on irradiation.

Table VI. Groups Used in Conjugates of Oligonucleotides

groups	refs
Fluorescent Dyes	
fluoresceins	99, 103, 113, 114, 146, 157, 160, 163, 168, 198, 200, 208–211
tetramethylrhodamine	99, 113, 131, 168, 209, 211
Texas red	99, 113
pyrene	109, 210
bimane•	130, 166, 192
mansyl	102
dansyl	109, 126, 191
proflavine	197
eosin	130, 163
naphthalene derivatives	123, 130
coumarin derivatives	130, 207
Intercalating Agents	
acridine	48, 49, 118, 134, 136, 149, 212–217
oxazolopyridocarbazole	177, 218
anthraquinone	135
phenanthridine	151
phenazine	219
Proteins	
peroxidases	99, 140, 176, 179, 220
IgG	179
alkaline phosphatases	99, 103, 121, 170
polylysine	130, 221–227
nucleases	147, 180, 228
Cross-Linking Agents	
alkylating agents	106, 119, 229–233
azidobenzenes	100, 163, 164, 190, 193, 196, 215
psoralen	112, 181, 182, 189, 205, 249
iodoacetamide	101
azidoproflavin	194
azidouracil	158, 159
platinum(II)	227, 234
Chain-Cleaving Agents	
EDTA/Fe^{II}	108, 173, 174, 236–238
phenanthroline/Cu^{II}	120, 150, 239, 240
porphyrin/Fe^{II}	241, 242
Others	
biotin	107, 109, 110, 122, 124, 126, 130, 133, 142, 143, 145, 148, 154–157, 162, 168, 169, 171, 195, 199, 200, 202, 210, 243
solid matrixes	125, 127, 128, 140, 170, 175, 244, 245
dinitrophenyl	109, 165
trinitrophenyl	200
proxyl spin-label	191
fluorene	203, 204
isoluminol	99
digoxigenin	246
puromycin	247
DTPA (chelating agent)	182
phospholipid	248
cholesterol	61, 307

Finally, attention is drawn to an old reaction that has not been used for conjugation but which is unusual in that it permits selective reaction at oligonucleotide hydroxyl groups when more often it is the bases that react most readily. This is the reaction of acetic anhydride in water (*206*). No reaction was found at the bases and mixed-anhydride formation at phosphates is readily reversed. Thus, in the case of 5′-phosphorylated oligodeoxynucleotides, reaction occurred only on the 3′-hydroxyl group.

D. Conjugate Groups. Many of the conjugate groups used with oligonucleotides are given in Table VI. This is not an exhaustive list of the use of these compounds, particularly of probes where the literature is extensive. Rather, it is intended to indicate the nature of conjugates studied and to serve as a source of the methods for coupling different groups.

Included are some recent, more specific ways for immobilizing oligonucleotides on solid supports, but the older literature on this subject is not covered and may be obtained from the references cited.

There is a very large amount of literature on chemically reactive oligonucleotides, particularly on those bearing nitrogen mustard type alkylating groups. Reference has been given to a review plus a few more recent examples. No attempt is made here to discuss the chemical reactions of these compounds.

A choice that has to be made for each conjugate group is the length of the spacer used to link it to the oligonucleotide. This is particularly important in the case of an intercalating agent that has to interact with the helix. Here, a very small change in spacer length can influence the outcome, but chains of five or six carbon atoms were best (*64, 177, 212, 213*). For psoralen to cross-link with the opposite strand, a shorter spacer of two carbons was required, and yields fell six-fold if this were increased (*249*). For biotin or fluorescent tags that do not interact with the helix, a longer spacer of eleven or twelve atoms was preferable to minimize steric inhibition of hybridization (*103, 127*).

4. PROPERTIES OF MODIFIED OLIGONUCLEOTIDES AND CONJUGATES

The development of conjugates with new properties should improve existing techniques and lead to the development of new uses and ideas. Advances are being made in the areas of automated sequencing (*113, 208*) and nonradioactive probes (*250*) and in accurate chemical cleaving of DNA and RNA (*101, 251–253*). Chemically reactive probes can be used to place a tag on a particular base within an RNA (*181*) and have been used to demonstrate parallel helix formation (*120, 194*). Nonradiative energy transfer between oligonucleotides can be used to indicate their separation in solution (*209, 220*). This seems a particularly promising technique that could be applied in many different ways and has already been used to investigate the structure of the Holiday junction (*211*). The same principle has been used to investigate interactions of oligonucleotides with proteins (*207*).

It is beyond the scope of the present review to cover all the uses and developments involving oligonucleotides. Rather, the emphasis here will be on the biological properties of modified oligonucleotides, in particular the effect of modification on hybridization, stability, and cell uptake. The final section discusses antisense inhibition, which is the most demanding use of oligonucleotides, requiring that they find and hybridize with their complementary sequences inside cells. Again, the approach taken here is to examine the general consequences of modification rather than the particulars of individual cases.

A. The Effect of Modification on Oligonucleotide Hybridization. Modifications to the internucleoside phosphates can affect hybridization in a number of different ways, but it is important that they should not prevent base pairing. Reduction in charge density lessens electrostatic repulsion between the strands and should facilitate their association. This effect will be greatest at low salt concentrations, where the shielding of the charges is least. Steric interactions of substituents will normally destabilize the helix as, it has been suggested, will their electronic and other effects (*254*). These might include disruption of hydration of the helix. However, the grooves of the hybrid might also provide a more lipophilic environment for the sequestration of hydrophobic substituents, thereby promoting hybridization. Cases where stronger hybridization resulted on increasing the lipophilicity of the substituent may be examples of such an effect (*43, 62*).

The relative contributions of all these factors are governed by external conditions such as salt concentration as well as intrinsic factors such as the length of the oligonucleotide, the degree of modification, the localization of a given modification relative to the ends or middle of the helix, and the sequence of bases around it (*254*). Thus, the consequences of a particular modification will vary from case to case. The complexity of this situation has prevented a clear understanding of the effects of phosphate modification on hybridization.

A complicating feature is the chirality of the phosphorus atom following modification. Absolute stereochemistry has been assigned by X-ray crystallography (*255*) and NMR (*256, 257*) and by enzymic (*258–261*) and chemical methods (*33, 44, 76*). A molecule with n chiral phosphorus atoms will consist of 2^n isomers, and one of the most challenging areas of oligonucleotide chemistry is the development of diastereospecific synthesis (*262–267*). Attempts to use diastereomerically pure starting materials in the usual synthetic approaches resulted in racemization (*53, 73, 268, 269*).

When small numbers of isomers are involved, resolution is possible by chromatography, and the pure diastereomers have been used for block condensation. This approach has been largely limited to the construction of short backbones with alternating unmodified and chiral phosphates or longer molecules with a single modification (*46, 48, 270–272*). Enzymatic synthesis of phosphorothioates gives the R_p isomer exclusively (*50, 51, 273*) and has enabled studies of polynucleotides with extensively modified, stereopure backbones (*274*). It is not yet possible to synthesize by chemical means diastereomerically pure chains of the length necessary for antisense inhibition.

In their early studies of uncharged methylphosphonates and triesters, Miller and Ts'o found that racemic di- to tetramers hybridized to unmodified strands with greater affinity than the parent phosphodiesters (*275, 276*). While diastereoisomers of dimers differed from each other, both formed more stable hybrids than the natural, charged compounds and were less effected by salt concentration. This was attributed to the lack of charge–charge repulsion between the strands of the complex (*17, 32*). An adverse effect on hybridization as the size of the substituent increased from PCH_3 to $POCH_3$ to $POCH_2CH_3$ seemed to be due to steric interactions.

Similar improvements in hybridization were reported for other low molecular weight triesters and phosphoramidates (*34, 43, 62*). With substituted amidates, these included a positively charged backbone. In some cases, however, bulkier substituents improved rather than diminished hybridization.

When a single, uncharged group is incorporated into an oligonucleotide, a different effect is seen and the stability of the helix is unchanged (*191, 271*) or reduced in most situations (*75, 254, 256, 277*). Possibly the removal of a single charge out of many makes little difference to the overall electrostatic repulsion between the strands and the other destabilizing effects of substitution gain in relative importance. The difference between the diastereomers is more pronounced with greater destabilization when the substituent points into the major groove rather than away from the helix (*75, 254, 256*).

With longer, extensively modified oligonucleotides, the complexity of the mixture becomes much greater and as each diastereomer in a pair is slightly different, some het-

erogeneity in overall properties is expected. An uncharged octamer of thymidine with an ethyl phosphotriester backbone could be separated into fractions with different affinities for poly(dA) (*40*). The real test of these compounds, however, is the effect of extensive substitution on the properties of a longer heterosequence.

The hybrid of a 20-mer containing 18 methylphosphonates melted only 4 °C below the fully charged duplex with little broadening of the melting curve in 0.1 M salt (*278*). In another study, a 15-mer containing 11 phosphonate linkages melted 5 °C higher than the diester in 0.015 M salt and 8 °C lower in 0.15 M salt (*64*). Heterogeneity in hybridization would be reflected in a broadening of the melting curve which was not appreciable in either of these examples. From the limited data presently available, it would appear that, in practice, the complexity of the diastereomeric mixture does not greatly reduce or broaden the spectrum of affinity for the target sequence of phosphonates when compared to that of phosphodiesters. That is not to say that higher melting would not result from the optimum, stereopure backbone.

At variance with these findings is the suggestion that methylphosphonates are inherently unsuitable for adopting a right-handed helical conformation so that melting temperature decrease to below 20 °C with chain lengths greater than four. Methyl triesters were not found to share this property and were proposed as superior antisense agents with high melting temperatures that are not influenced by the chirality of the phosphorus (*36, 279*). However, they hybridize poorly with RNA, which is the usual target for antisense inhibition (*280*).

Extensive substitution of longer oligonucleotides with various phosphoramidate linkages gave somewhat less stable hybrids than the phosphonates. Those derived from primary amines were superior to those from secondary amines (*64*). Cationic amidates showed a reversal in salt dependence and formed hybrids that were more stable in low salt then phosphodiesters but less stable in high salt (*63*).

A common finding in many studies of noncharged phosphate modifications is that hybridization to RNA is less efficient than that to DNA (*40, 43 48, 63, 86, 174, 270, 280*). It has been suggested in the case of ethyl phosphotriesters that this results from the inability to form an A type helix, where the loss of rotational freedom of the ethyl group is greater (*40*).

With a single phosphorothioate linkage, only the R_p isomer with sulfur pointing into the major groove destabilized the helix (*281*). Multiple phosphorothioate linkages, either all R_p or a racemic mixture, lowered the melting temperature by an amount that depended on the base composition of the oligonucleotide but was at least 7 °C for a 15-mer (*56, 134, 274*).

Modifications other than at internucleoside phosphate may also affect hybridization. α-Oligonucleotides, for example, hybridize with β-RNA and DNA by forming the usual Watson–Crick base pairs. In fact, these hybrids are considerably more stable than when both strands are β (*243*). Unlike natural duplexes, however, those with an α-chain have parallel strands (*215, 216, 282, 283*). An exception is the complex between α-oligothymidylic acid and β-poly(rA), which is antiparallel (*239*). This particular α-oligonucleotide is also unusual in that it can form a double-stranded helix with itself containing T·T base pairs (*284*). Similar parallel self-pairing was observed on neutralizing the phosphates of β-oligothymidylate as methyl triesters (*42*).

In an attempt to stabilize hybridization with very short oligonucleotides, Letsinger and Schott attached an intercalating agent to the phosphate group of TpT (*151*). This proved successful and, as discussed later, permits the use of shorter oligonucleotides than would otherwise be possible for antisense studies. The physical chemistry of these interactions has been investigated (*165, 212, 213, 285*) and it has been shown that an intercalating agent at the end of an oligonucleotide, especially the 3′-end, is more beneficial than on an internucleotide phosphate and that a second intercalating group offers no further advantage (*136, 213*).

α-Oligonucleotides and others with methylphosphonate or phosphotriester linkages also benefited from the addition of an intercalator (*48, 64, 177, 215, 218*). In these conjugates, two different mechanisms for stabilizing the duplex are combined. The only intercalator that has been combined with a phosphorothioate backbone was of somewhat uncertain efficacy (*134*). No stabilization was observed and, if confirmed in other cases, this may reflect steric effects of the larger sulfur atom at the site of intercalation.

B. The Effect of Modification on Nuclease Resistance. A number of studies have demonstrated degradation of unmodified oligonucleotides at greatly varying rates in different cells or in the serum-containing media used for cell culture. Survival times vary from minutes to days (*56, 286–293*). Consequently, it has been a goal in many approaches, particularly in the antisense field, to develop nuclease-resistant derivatives on the assumption that these will be more potent. Of course, there is the chance that they will also be more toxic because they survive longer.

It was recognized early on that the rate of degradation of RNA by exonucleases from snake venom and spleen was slowed considerably by phosphorothioate groups (*294*). The former enzyme can cleave the R_p diastereomer but not the S_p or adjacent R_pS_p or S_pS_p groups (*53, 262, 295–297*). In contrast, nucleases S1 and P1 are specific for the S_p isomer (*53, 297, 298*) while ribonucleases A and T2 do not distinguish between different configurations (*296*). With DNAse I, sequence or base composition also effect the rate of digestion of phosphorothioate-substituted polynucleotides (*274*).

As a result, oligodeoxynucleotides with a high proportion of unresolved phosphorothioate groups are almost totally resistant to snake venom phosphodiesterase, are degraded 2–45 times more slowly than normal by S1 and P1 nucleases, and survive many times longer than unsubstituted oligonucleotides in human serum (*56*).

All other modifications to internucleoside phosphates that have been investigated also inhibit the action of nucleases. Most reports suggest complete resistance of phosphoramidate (*43, 45, 58, 62*), phosphonate (*18, 270*), or phosphotriester (*32, 43, 299*) linkages toward the nucleases that have been tested. An exception is the very slow rate of cleavage by snake venom or spleen phosphodiesterases of unsubstituted phosphoramidate observed for the sequence d(ApA) but not for TpT (*43, 65*). All N-substituted phosphoramidates were resistant. An early report of slow cleavage of one of the diastereomers of phosphonates by snake venom phosphodiesterase could not be confirmed (*18, 270*).

While the modified linkages themselves may be resistant, in oligonucleotides containing mixtures of modified and natural linkages, exonucleases that work progressively from one end of the chain can sometimes skip over an isolated phosphonate or triester linkage to cleave

the adjacent phosphodiester at a reduced rate (*27, 270, 300*). This may be more difficult if the phosphonate is near the end of the chain and the presence of two adjacent internal methylphosphonates in oligothymidylic acid blocked the progress of the enzyme much more effectively than just one (*27, 300*). Similarly, blocks of contiguous phosphorothioates at the ends of the chain gave resistance to exonucleases while conserving desirable properties of the unmodified backbone in between (*56*). Resistance to endonucleases may be improved by reducing runs of contiguous phosphodiesters, particularly below four (*300*).

Modifications at groups other than phosphate may also induce resistance to nucleases. α-Oligodeoxynucleotides, for example, proved far more stable than β-oligodeoxynucleotides in a number of different biological environments (*291, 301*). Also, the presence of a bulky group such as an intercalating agent or even methylthiophosphate at the appropriate end of an oligonucleotide can preempt exonuclease attack (*134, 237, 291*).

RNA was found to be more stable than DNA in nuclear cell extracts (*302*). Methylation of the 2′-hydroxyl group increases its resistance to nucleases (*303*).

Nucleases are not the only enzymes involved in the catabolism of oligonucleotides inside cells. In cultured fibroblasts, oligonucleoside ethyl phosphotriesters were broken down quite rapidly, probably following deethylation. Methylphosphonates survived much better but were slowly degraded by a pathway that may begin with deglycosylation (*304*).

C. Cellular Uptake of Modified Oligonucleotides. A problem common to uptake studies is the difficulty in distinguishing material inside the cell from that bound to the outer membrane. Few studies have managed to do this convincingly enough to eliminate doubt. Consequently, confidence in the literature concerning this subject is not as high as is desirable.

Despite their high charge density, oligonucleotides are taken up reasonably well by mammalian cells. This appears to be an energy-requiring process and may involve receptor proteins on the cell surface. Intracellular concentrations may rise to about 10% of those outside the cell within 15 min to 2 h (*286, 290, 305, 306*). Shorter oligonucleotides are taken up somewhat more rapidly and phosphorothioates are taken up more slowly than unmodified oligonucleotides (*134*). Lipophilic substituents such as intercalating agents or cholesterol facilitate uptake (*216, 237, 307*).

Uptake of uncharged methylphosphonates appears to be quite different. Intracellular levels of dimers to nonamers reached extracellular concentrations within 1.5 h (*276*). This would appear to be passive diffusion across the cell membrane. Prokaryotes have not been investigated extensively, but in contrast, *E. coli* cells were impermeable to chain lengths greater than four (*308*).

D. Modified Antisense Oligonucleotides. Oligonucleotides complementary to strategic regions of viral or messenger RNA's were first shown by Zamecnik and Stephenson to inhibit viral replication (*309*). The structures of these highly specific, biologically active compounds can be predicted from the sequence of the target RNA and are therefore useful for genetic analysis and attractive candidates for therapeutic agents. As they generally prevent expression of the sense strand, they have become known as antisense oligonucleotides. The object of this section is not to review the antisense approach or to discuss strategies for its use, as this has been done elsewhere (*310, 311*). Rather, attention will be focused on the effects of chemical modification on activity.

Comparatively few true conjugates have been used for antisense studies so far although modifications to the backbone have been used extensively. These will play an increasingly important role in designing the next generation of compounds where components with particular properties will be required.

The factors that are usually assumed to limit the activity of antisense oligonucleotides are cellular uptake, resistance to nucleases, and the stability of the hybrid formed. Modifications are usually chosen to improve one or more of these properties, as discussed in the previous sections. Overall activity results from the interplay of these and other factors whose relative importance is generally not known. However, a steady improvement in activity has been achieved by using this rational approach that is encouraging for the future of designing conjugates to meet specified requirements.

The modifications to the backbone that have been used most extensively are those discussed in previous contexts: phosphorothioates, methylphosphonates, phosphoramidates, and phosphotriesters. Chimeric oligonucleotides with several modified linkages at each end have been more successful with phosphorothioates than methylphosphonates (*28, 60, 278*).

A fundamental difference between negatively charged phosphodiesters or phosphorothioates and the uncharged derivatives is their acceptance by ribonuclease H. This enzyme degrades the RNA strand of an RNA/DNA duplex and has been shown to be an important factor for the activity of antisense oligonucleotides in a number of systems. In these cases, binding of oligonucleotide at any site on the RNA should lead to cleavage and irreversible inactivation. In situations where this enzyme is not available, oligonucleotides are thought to inhibit expression by passive steric blocking of translation or other events (hybridization arrest). The particular binding site is then of great importance. With mRNA, for example, it appears that oligonucleotides can be readily displaced by ribosomes and were only effective when bound to the 5′-capped end or, to a lesser extent, across the AUG initiator (*312*).

Ribonuclease H recognizes the charged, unmodified or phosphorothioate backbone in oligodeoxynucleotides but not the uncharged methylphosphonate or phosphoramidates (*56, 249, 252, 300, 313–315*). Thus, passive hybridization arrest is the only known mechanism open to the latter compounds and the target site may be of prime importance with these modifications. There is a suggestion, however, that methylphosphonates may be more resistant to displacement from the RNA by cellular factors which could be a property of the uncharged backbone and could improve activity by the passive hybridization mechanism (*316*).

The importance of the binding site is well illustrated by the α-deoxyoligonucleotides which, despite their good hybridizing ability, do not activate ribonuclease H and were found inactive as antisense agents except when complementary to the 5′-capped end of mRNA (*223, 314, 317–320*).

With chain lengths of 20, methylphosphonates, phosphorothioates, and phosphonamidates were found to reduce the concentration of oligonucleotide necessary for good viral inhibition from over 20 μM to 5 μM or less (*58, 278*). Perhaps because of their somewhat weaker hybridization, reducing their chain length to 15 reduced activity far more than with the unmodified series. [In another study, however, 15 was found to be the optimum chain length for phosphonates (*331*).] Methylphosphonates with

chain lengths of 10 or less may have to be used at concentrations of 100 μM or higher to achieve good results. Even so, they are often more active than the unmodified compounds (*276, 308, 316, 321–326*). Inappropriate chain length or binding site might be among the reasons for poor or no activity found in other examples (*28, 224, 314, 315, 327*).

In the ribo series, both methylation of the 2′-hydroxyl group and phosphorothioate substitution were necessary for antiviral activity in one study (*60*). The 2′-*O*-methyl substituent alone does not permit ribonuclease H digestion of the complementary RNA (*328*).

With chemical modification, there is always the possibility of introducing unwanted biological properties. This was seen with the phosphorothioates, which can bind rather well to a number of proteins and inhibit certain enzymes (*314, 329, 330*). As a result, in some antiviral and other assays, inhibition was not restricted to antisense sequences (*58, 61, 327, 331*). In further studies, antisense effects were separated from other effects of phosphorothioates (*332, 333*).

Among the true conjugates are derivaties with acridine linked at the 3′-end to stabilize hybridization. This modification increases activity and permits the use of unusually short oligonucleotides (*334–337*).

Conjugates of polylysine were reported to lower the concentration necessary for good antiviral activity to 1 μM or below (*59, 221, 224–226*). This more than compensates for the increase in mass of material required due to the doubling of molecular weight (the preferred molecular weight for the peptide is about the same as that of a 20-base oligonucleotide). This approach does not work in all cells and polylysine is toxic at higher concentrations (*225*).

Another group that, like polylysine, is intended to increase uptake is cholesterol. This has beneficial effects for both unmodified and phosphorothioate backbones as well as for alkylating derivatives (*61, 307*).

In 1967, Belikova et al. made a dinucleotide carrying an alkylating reagent for the modification of complementary sequences (*119*). This was probably the first example of what would now be called an antisense oligonucleotide. Reactive compounds of this type have been investigated extensively by groups in the USSR (*229*). In recent years, attention in the West also has turned to these and other derivatives in Table VI that can cross-link or cleave the target RNA or DNA. This has come about with the need to develop more potent derivatives to improve the potential for therapeutic applications. If hybridization is reversible, so one argument goes, then a higher concentration of the oligonucleotide is required in the cell to maintain the complex than if the process were not reversible. Hence oligonucleotides that irreversibly change the target may be more potent.

This was demonstrated using antisense oligonucleotides to inhibit replication of a single-stranded DNA by *E. coli* DNA polymerase I (*227*). The enzyme was not inhibited by an unmodified oligonucleotide hybridized to the template unless the two were cross-linked. Presumably, as the enzyme reads the DNA, it can displace hybridized oligonucleotide in its path but becomes stalled when the oligonucleotide is irreversibly bound to the template.

Despite the large body of chemical work in this area, there are only a few examples of the use of chemically reactive oligonucleotides as antisense agents either in vitro or in vivo. These include an early inhibition of IgG synthesis in cells (*229*). More recently, the photoactivated cross-linker psoralen was attached to oligonucleoside methylphosphonates and found to increase their potency by 20–40-fold in antiviral or inhibition of translation assays (*249, 326*).

A concern in using reactive conjugates of this type is the possibility of nonspecific reaction with other cell components leading to toxicity or, alternatively, self-inactivation. The latter has been found to limit the use of EDTA attached to methylphosphonates due to autocleavage of the conjugate group by the free radicals it generates (*174*).

5. CONCLUDING COMMENTS

Much current research at the interface of biology and chemistry is directed at understanding and predicting the effect of molecular structure on biological activity. Oligonucleotides are particularly well-suited for this type of activity. This is because of the nature of their site of action. Unlike most active compounds, this is not some hydrophobic pocket on a protein with unique and unpredictable properties. Rather it is a nucleic acid whose precise sequence can be determined and whose interaction with the oligonucleotide can be predicted with some confidence.

The encouraging finding from the work reviewed here is just how robust this mechanism for base pairing is toward chemical modification. The nature of the backbone can be changed from anionic to uncharged or cationic, from hydrophilic to lipophilic without seriously interfering with hybridization. Attempts to improve antisense inhibition in a rational way by altering specific features of the molecule have successfully increased activity. This is promising for prospects of tailoring molecules to particular purposes. These include improving performance and utility in areas where oligonucleotides have already found application such as diagnosis, genetic analysis, automated sequencing, and many others. It also includes meeting the pharmacological requirements for possible future drug development.

Whether or not modified oligonucleotides and their conjugates have the necessary attributes for pharmaceutical use, it is clear that they provide an unusual opportunity for the rational design of useful molecules with specific properties.

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TEACHING EDITORIAL

A Brief Guide to Nucleic Acid Chemistry

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STRUCTURE OF NUCLEIC ACIDS

Nucleic acids encode the genetic information of all living organisms and are intimately involved in the conversion of this information into cellular proteins and enzymes. Cellular nucleic acids are polymeric molecules which are composed of three basic units: a base, a sugar, and a phosphodiester group. The arrangement of these three groups to form either DNA or RNA is shown in Figure 1.

The nitrogenous, heterocyclic bases are derivatives of purine or pyrimidine. The four bases commonly found in DNA are adenine, guanine, cytosine, and thymine. These same bases are also found in RNA with the exception that thymine is replaced by uracil. In addition, a variety of modified bases, such as N^4,N^4-dimethyladenine and N^7-methylguanine are found in messenger RNA, transfer RNA, and ribosomal RNA.

The bases are linked to 2′-deoxyribose in DNA or ribose in RNA via an *N*-glycosyl bond to form a nucleoside. The nucleosides are named according to the heterocyclic base which they contain. The nucleosides found in DNA are 2′-deoxyadenosine, 2′-deoxyguanosine, 2′-deoxycytidine, and thymidine. The nucleosides commonly found in RNA are adenosine, guanosine, cytidine, and uridine. 2′-*O*-Methylribosyl nucleosides are also found in RNA, particularly in messenger RNA and ribosomal RNA.

The nucleosides are linked together via phosphate ester groups to form the sugar phosphate backbone of the nucleic acid. Thus esterification of the 3′-hydroxyl of one nucleoside and the 5′-hydroxyl of the next nucleoside unit results in the formation of a 3′→5′ internucleotide bond. This type of linkage is found in both DNA and RNA. In the case of RNA the internucleotide bond could also extend from the 2′-hydroxyl to form a 2′→5′ internucleotide bond. Such 2′→5′ linkages are found in certain oligoadenylates which are synthesized in mammalian cells in response to interferon (*1*).

A single phosphorlyated nucleoside unit is called a nucleotide. The sequence of nucleotides within the nucleic acid chain determines the genetic information encoded by the nucleic acid. The purine bases, adenine and guanine, can form hydrogen bonds with the pyrimidine bases, thymine (uracil in RNA) and cytosine, respectively (see Figure 2). The base pairs formed between these so-called complementary bases enable separate chains of nucleic acids to interact with one another. In DNA, separate nucleic acid strands form a double-helical structure in which the sugar phosphate backbones run in an antiparallel direction. Double-helical DNA, which usually exists in a right-handed, B-type conformation, can exist in a variety of conformational forms including left-handed helices (*2*). The particular conformation depends upon the nucleotide sequence and the environment of the DNA. DNA can also exist in a triple-stranded form in which three bases form a triad via hydrogen-bonding interactions as shown in Figure 2 (*2*, *3*).

Although RNA is often thought of as a single-stranded molecule, self-complementary nucleotide sequences present within the single strand give rise to the formation of intramolecular helical regions. These intramolecular interactions can produce a tremendous variety of helical and looped structural regions and account for the secondary structure within RNA molecules. In addition to these secondary structural features, further folding and hydrogen-bonding interactions between bases in remote parts of the molecule give rise to a tertiary structure. The combination of these interactions results in overall three dimensional structure, whose complexity approaches that found in proteins. This complexity has been most clearly revealed in the structure of transfer RNA (*4*).

Nucleic acid structure has been elucidated at the atomic level of resolution by nuclear magnetic resonance spectroscopy and X-ray diffraction techniques. In addition to studying nucleic acid structure, recent X-ray experiments have been used to examine the interactions of proteins with nucleic acids. For example, the structures of the complex formed between the restriction enzyme *Eco*R I and a deoxyribonucleotide duplex, and of the complex formed by glutaminyl tRNA with its cognate aminoacyl synthetase have been determined (*5*, *6*). Such studies promise to lead to further insights into how nucleic acids